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A Class of Benzenoid Chemicals Suppresses Apoptosis in *C. elegans*

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Benzene is a human carcinogen that might act through both genotoxic and nongenotoxic mechanisms to promote tumorigenesis. The genotoxic effects of benzene are well established, however, its potential nongenotoxic roles in carcinogenesis are poorly understood. We find that benzene suppresses somatic apoptosis in C. elegans*; this suggests a potential nongenotoxic mechanism by which this chemical might promote tumorigenesis. We find that two other benzenoid chemicals, biphenyl and toluene, also inhibit apoptosis in* C. elegans*. Notably, these chemicals are suspected carcinogens in mammals; this suggests that a subclass of benzenoid chemicals might promote tumorigenesis by suppressing apoptosis. A benzene metabolite, 1,4-benzoquinone, can directly inhibit the activity of caspase-3; this suggests a general molecular mechanism by which benzenoid chemicals might suppress apoptosis. These findings suggest that* C. elegans *is an excellent alternative animal model for studying the antiapoptotic activity of tumor-promoting chemicals and for identifying in vivo targets of these chemicals.*

Introduction

Chemical carcinogens contribute to tumorigenesis in two different ways. Genotoxic carcinogens cause cancer by directly damaging DNA and thus cause mutations in tumor-suppressor genes and proto-oncogenes.[1,2] By contrast, nongenotoxic carcinogens are thought to promote the survival of latent tumor cells through various different mechanisms that do not involve direct DNA damage.[3] One way that nongenotoxic carcinogens could promote tumorigenesis is by suppressing apoptosis, which removes cells that are at risk of becoming cancerous.[4] Indeed, two nongenotoxic carcinogens, naphthalene and *para*-dichlorobenzene (PDCB), were recently found to suppress apoptosis in *C. elegans*.[5] Since both naphthalene and PDCB are small aromatic hydrocarbons, it is an interesting possibility that other aromatic chemicals with benzene-like structures might also have apoptosis inhibitory activity.

Benzene, biphenyl, and toluene are ubiquitous environmental pollutants and potential carcinogens that are widely used in industry and in commercial products. Benzene is a recognized carcinogen that causes various leukemias in humans.[6–8] Biphenyl derivatives, such as polychlorinated biphenyls (PCBs), are "reasonably anticipated to be human carcinogens" as defined by the National Toxicology Program (NTP).[9] Although toluene is considered "not classifiable" regarding human carcinogenicity,[9] some reports suggest that it might promote tumorigenesis.[10,11]

Many standard assays have been developed to evaluate the genotoxicity of suspected carcinogens.[12] By contrast, very few assays currently exist to evaluate nongenotoxic carcinogens;[3] the standard assay is the two-year rodent bioassay, which is both costly and time consuming. Therefore, development of an alternative animal model that allows rapid evaluation of nongenotoxic carcinogens is an important issue.

C. elegans is an excellent animal model for investigating the basic mechanisms of apoptosis. Of the 1090 somatic cells born during the development of *C. elegans* hermaphrodites, 131 invariably undergo apoptosis.[13,14] Unlike mammalian cells, which are prone to undergo apoptosis in response to genotoxic stress, *C. elegans* somatic cells are resistant to genotoxic insult.[15,16] Therefore, it is easier to interpret phenotypes caused by suspected nongenotoxic carcinogens in *C. elegans* than it is in mammals. Apoptosis regulation is less complex in *C. elegans* than in mammals, but key apoptotic genes are conserved between nematodes and humans.[17] In both, activation of proapoptotic EGL-1/BH3-only proteins initiates apoptosis by binding to and inhibiting the activities of CED-9/Bcl-2 family members. This leads to the activation of CED-4/Apaf-1, which in turn activates the CED-3/caspases, the key enzymatic executioners of apoptosis.[18] The high level of conservation of key components of apoptosis between nematodes and humans indicates that insights derived from the studies of apoptosis in *C. elegans* are likely to be highly relevant to the understanding of apoptosis regulation in humans.

Recently, a novel, oil-based method was developed to facilitate the delivery of hydrophobic chemicals into *C. elegans* and led to the identification of naphthalene and PDCB as the first small-molecule apoptosis inhibitors in *C. elegans*.[5] This method, in combination with the ease of assaying apoptosis in *C. elegans* and the availability of powerful genetic methods and biochemical assays for cell death, makes *C. elegans* an excellent animal model for studying the biological activities of nongenotoxic carcinogens.

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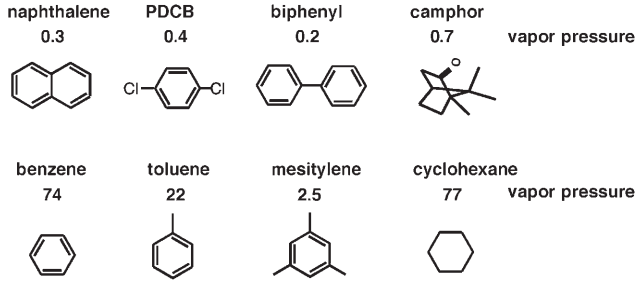
Results

A subgroup of benzenoid chemicals suppresses apoptosis in *C. elegans*

In *C. elegans* the identity of every somatic cell is invariant from animal to animal. As a result, it is possible to quantify the cell-death defects of different *C. elegans* strains simply by counting the number of cells that should have died during embryogenesis but inappropriately survive. Normally 16 cells in the anterior pharynx of wild-type (N2) *C. elegans* animals undergo apoptosis during embryonic development. Mutations in genes that regulate apoptosis can cause some or all of these cells to survive inappropriately. For example, in the *ced-3*(*n2438*) mutant, which carries a partial loss-of-function mutation in the *ced-3* gene, an average of 1.5 extra undead cells are seen in the anterior pharynx.[5] Chemicals that suppress apoptosis can substantially increase the number of inappropriately surviving cells in these animals. For example, two nongenotoxic carcinogens, naphthalene and PDCB, were recently found to suppress apoptosis in *C. elegans*.[5] Naphthalene and PDCB have similar physical properties, but appear structurally quite different (Scheme 1). To investigate the common property that might



Scheme 1. Chemical structures of the compounds tested in this study and their respective vapor pressures.

underlie the antiapoptotic activity of these two chemicals, we tested whether other chemicals with either similar physical or chemical properties suppress apoptosis in *C. elegans* by counting the number of extra cells in the anterior pharynx of *ced-3*(*n2438*) animals exposed to different chemicals using the oil-based chemical delivery protocol.[5]

Biphenyl and camphor have physical properties similar to those of naphthalene and PDCB. These are all hydrophobic white solids that sublimate directly from the solid phase to the gas phase but are structurally quite different (Scheme 1). We found that camphor does not suppress apoptosis in *C. elegans*, whereas biphenyl does (Figure 1). Since a benzene ring is the common structural moiety shared by the three apoptosis-inhibitory chemicals (naphthalene, PDCB, and biphenyl), we tested whether other benzenoid chemicals inhibit apoptosis in *C. elegans*. Indeed, benzene and toluene, which are clear volatile liquids, also suppresses apoptosis in *C. elegans* (Scheme 1, Figure 1), indicating that benzene might be the basic structural unit that can result in suppression of apoptosis in animals. Consistent with this notion, cyclohexane, which differs from

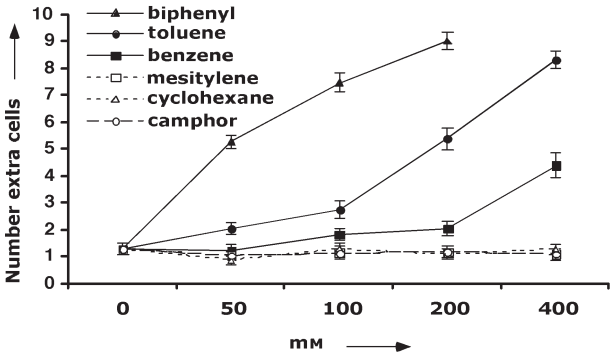


Figure 1. Biphenyl, toluene, and benzene inhibit apoptosis in *C. elegans* in a dosage-dependent manner. *ced-3*(*n2438*) animals were exposed to biphenyl, toluene, benzene, cyclohexane, camphor, or mesitylene dissolved in soybean oil. The *y*-axis represents the average number of inappropriately surviving cells in the anterior pharynx of *ced-3*(*n2438*) animals. Error bars show standard errors of the mean. Twenty animals were scored for each chemical treatment.

benzene by the lack of double bonds and thus lacks delocalized electrons, fails to inhibit apoptosis in *C. elegans* at all concentrations tested. Interestingly, mesitylene, which resembles toluene in structure but contains two additional methyl groups, also fails to inhibit apoptosis. These two additional methyl groups might prevent the formation of reactive metabolites important for apoptosis inhibition (see below). Taken together, these results suggest that benzene might be the simplest member of the benzenoid chemicals that can suppress apoptosis in *C. elegans*.

When benzenoid chemicals are dissolved in oil, relatively high concentrations are needed to suppress apoptosis in *C. elegans* (Figure 1). However, much lower gaseous concentrations of these chemicals cause a similar level of apoptosis suppression. For example, less than 4 μM of gaseous biphenyl or naphthalene can result in greater cell death inhibition than 100 mM of biphenyl or naphthalene in oil (Figure 2). Since these are the

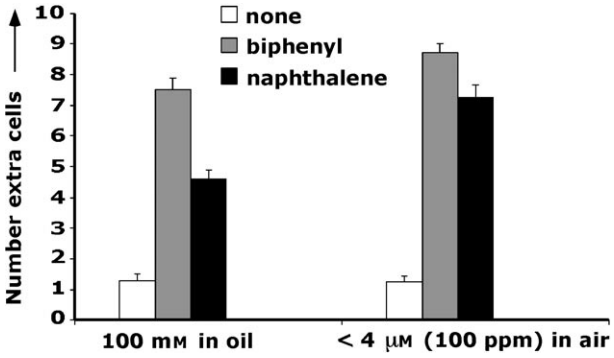


Figure 2. The apoptosis inhibitory activity of biphenyl or naphthalene differs in different solvents. *C. elegans* animals (strain *ced-3*(*n2438*)) were exposed to different concentrations of biphenyl and naphthalene in air or oil as indicated, and the numbers of extra undead cells were scored. Based on the vapor pressure, the maximum concentration of biphenyl or naphthalene in air is ~4 μM. The *y*-axis represents the average number of inappropriately surviving cells in the anterior pharynx of *ced-3*(*n2438*) animals. Error bars show standard errors of the mean. Twenty animals were scored for each chemical treatment.

gaseous concentrations that humans would normally encounter when using these chemicals, the observed apoptosis-inhibitory activity of these chemicals is of great physiological relevance. However, suppression of apoptosis by gaseous naphthalene or biphenyl is less consistent and does not respond in a dosage-dependent manner due to the difficulty of working with gas (data not shown).[5] Therefore, the more consistent and reproducible oil-based assay was adopted in our study to quantify the effects of these chemicals on worms.

Biphenyl, toluene, and benzene are general inhibitors of apoptosis

To examine whether biphenyl, toluene, and benzene are general inhibitors of apoptosis, we investigated the effects of these chemicals on apoptosis in other cell death mutant backgrounds. These chemicals also increase the number of extra cells in *ced-4*(*n2273*) mutants (Figure 3 A). Like *ced-3*(*n2438*) mutants, these animals are partially defective in cell death, thus suggesting that the apoptosis-inhibitory activities of these chemicals do not depend on mutations in a specific cell-death gene. Importantly, benzene, toluene, and biphenyl do not increase the number of extra cells seen in *ced-3*(*n2433*), a strong loss-of-function *ced-3* mutant; this indicates that inhibition of apoptosis, rather than transformation of cell fates, causes the increase in extra cells. Interestingly, these chemicals do not seem to cause inappropriately surviving cells in wild-type animals; this suggests that these chemicals have greater apoptosis inhibitory activity on animals in which apoptosis is already compromised.

We next examined how benzene, toluene, and biphenyl affect apoptosis in wild-type animals by counting the number of cell corpses at different stages of embryogenesis. This is when most apoptosis occurs, and apoptotic cell corpses can be easily identified under Nomarski optics by their characteristic refractive appearance.[14] Benzene, toluene, and biphenyl significantly reduce the number of cell corpses observed in chemically treated wild-type embryos (Figure 3 B); this suggests that these chemicals generally suppress apoptosis during embryogenesis.

All these chemicals have some nonspecific toxic effects on *C. elegans*. They slow down animal development, reduce brood size, and cause some lethality at the same concentrations that affect apoptosis; however, chemically exposed animals appear anatomically and behaviorally normal (data not shown).

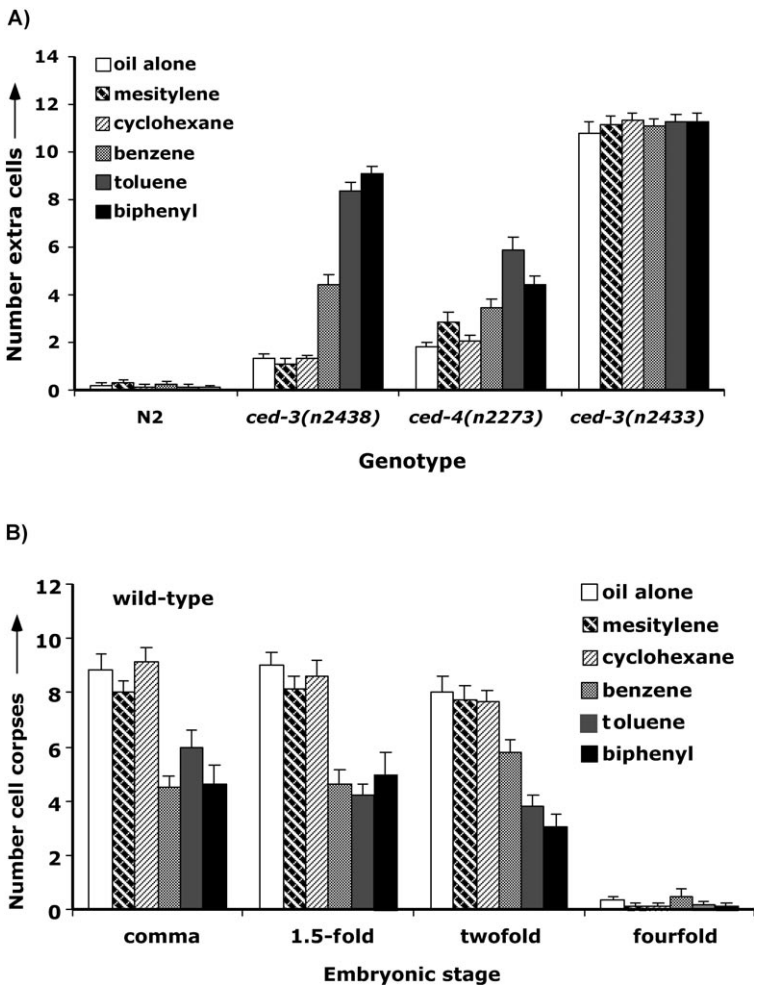


Figure 3. Biphenyl, toluene, and benzene generally suppress apoptosis in *C. elegans*. Animals with the indicated genotype were exposed to oil alone, biphenyl, toluene, benzene, cyclohexane, or mesitylene dissolved in soybean oil. A) Biphenyl, toluene, and benzene inhibit apoptosis in different genetic backgrounds. The numbers of extra cells in the anterior pharynges of L3 or L4 larvae were counted by using Nomarski optics (*y*-axis). Twenty animals were scored in each experiment. B) Biphenyl, toluene, and benzene reduce apoptotic cell corpses in wild-type animals. Cell corpses were counted in N2 (wild-type) animals treated with different chemicals. Embryos were scored at the comma, 1.5-fold, twofold, and fourfold stages. The *y*-axis represents the average number of cell corpses scored in the head region of embryos. Error bars show the standard errors of the mean. Fifteen embryos were scored at every embryonic stage in each experiment.

Biphenyl and toluene inhibit apoptosis induced by the activated CED-3 caspase

Naphthalene appears to inhibit apoptosis in *C. elegans* by inactivating the CED-3 caspase through one of its metabolites, 1,4-naphthoquinone.[5] To investigate whether biphenyl, toluene, and benzene might also directly target the CED-3 caspase, we tested whether these chemicals are able to block cell killing caused by ectopic expression of an activated version of the CED-3 protease (acCED-3) in the absence of its upstream activator, CED-4.[19] We used the *egl-1* gene promoter, a *C. elegans* dying-cell-specific promoter, to express acCED-3 in *ced-1*(*e1735*); *ced-4*(*n1162*) mutant animals that carry strong loss-of-function mutations in both the *ced-1* and the *ced-4* genes. Since CED-1 is required for the engulfment of cell corpses, apoptotic cell corpses persist in *ced-1*(*e1735*) mutants, and the death-inducing activity of acCED-3 can be easily quantified. CED-4 is required to activate the CED-3 zymogen,[20] so virtually no apoptosis occurs in *ced-4*(*n1162*) mutants[21] and very few cell corpses are seen in *ced-1*(*e1735*); *ced-4*(*n1162*) animals. By

contrast, in *ced-1*(*e1735*); *ced-4*(*n1162*) animals carrying an integrated P_{egl-1}acCED-3 transgene (*smIs111*), an average of 27 unengulfed cell corpses are seen at the fourfold embryonic stage (Figure 4). To determine if biphenyl, toluene, and benzene inhibit cell killing induced by acCED-3, we counted the number of cell corpses in animals with and without chemical treatment and found that biphenyl and toluene substantially reduced the number of cell corpses in *ced-1*(*e1735*); *ced-4*(*n1162*); *smIs111* transgenic embryos. This result suggests that biphenyl and toluene might directly act on acCED-3 (or its downstream effectors) to suppress apoptosis, independently of the CED-3 upstream activators. Benzene does not reduce the number of persistent cell corpses in this assay; this suggests that the apoptosis inhibitory activity of benzene might be too weak to inhibit cell killing induced by overexpression of acCED-3.

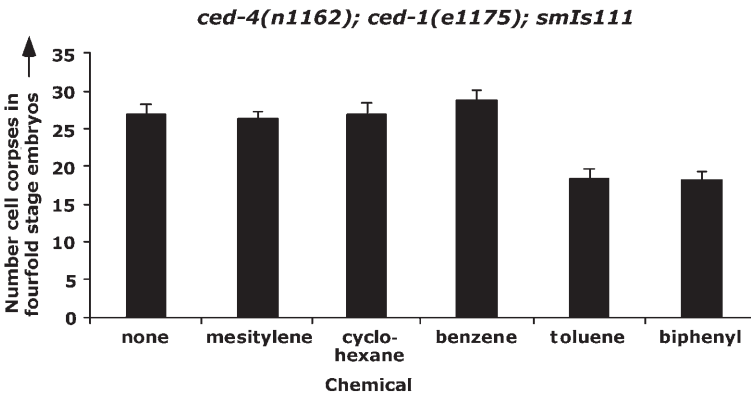


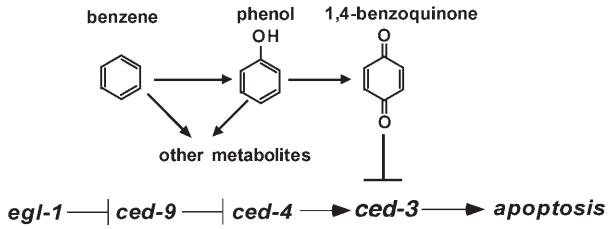
Figure 4. Biphenyl and toluene suppress activated CED-3 (acCED-3) induced apoptosis in the absence of CED-4. The *y*-axis represents the average number of cell corpses observed in the fourfold stage *ced-1*(*e1735*); *ced-4*(*n1162*) transgenic embryos containing the integrated P_{egl-1}acCED-3 transgene (*smIs111*) treated with the indicated chemicals. Fifteen embryos were scored for each condition. Error bars show standard errors of the mean.

1,4-Benzoquinone and toluquinone inhibit the activity of caspase-3 in vitro

A reactive naphthalene metabolite, 1,4-naphthoquinone (NQ), directly inactivates caspases by oxidizing specifically the catalytic cysteine residue in these proteases.[5] We therefore investigated the possibility that biphenyl, toluene, and benzene also suppress apoptosis in *C. elegans* through similar reactive quinone intermediates. Biphenyl metabolism is not well understood, whereas the metabolism of benzene and toluene has been better studied. Benzene metabolism is known to generate benzoquione (BQ) via a phenol intermediate (Scheme 2).[22,23] Toluene can be metabolized to generate cresol,[24] which is further metabolized to toluquinone (TQ).[25] We thus investigated whether biphenyl, toluene, benzene, phenol, BQ, and TQ inhibit the activity of human caspase-3 in vitro. We used the fluorogenic caspase-3 substrate, Ac-DEVD-AMC (7-amino-4-methylcoumarin), to monitor human caspase-3 activity in the presence or absence of these chemicals. Of the chemicals tested, only BQ and TQ inactivated caspase-3 (Figure 5); this suggests that, like naphthalene, benzene and toluene might inhibit apoptosis in *C. elegans* through reactive quinone metabolites. Interestingly, the three methyl groups in mesitylene would block the formation of quinone intermediates and thus the apoptosis inhibitory activity; and indeed, mesitylene fails to inhibit apoptosis in *C. elegans* (Figure 1). Taken together, these results support the hypothesis that benzenoid chemicals suppress apoptosis in animals through reactive quinone metabolites.

Discussion

In this paper, we investigate chemical inhibition of apoptosis in *C. elegans*. Unlike the case with mammals, environmental chemicals and pollutants have not been known to affect developmental apoptosis in *C. elegans*. Recently, we described the first chemical inhibitors of apoptosis in *C. elegans*.[5] Here, we extend our study to describe a class of related benzenoid chemicals that also suppress apoptosis in this



Scheme 2. Benzenoid chemicals might inhibit apoptosis through quinone metabolites. One of the major benzene metabolic products is phenol, which is either excreted or further metabolized into reactive 1,4-benzoquinone. Benzene also generates other metabolic products that are not shown here. The *C. elegans* central cell-death pathway that contains cell-killing genes *egl-1*, *ced-4*, and *ced-3* and the cell-death inhibitor *ced-9* is also shown.

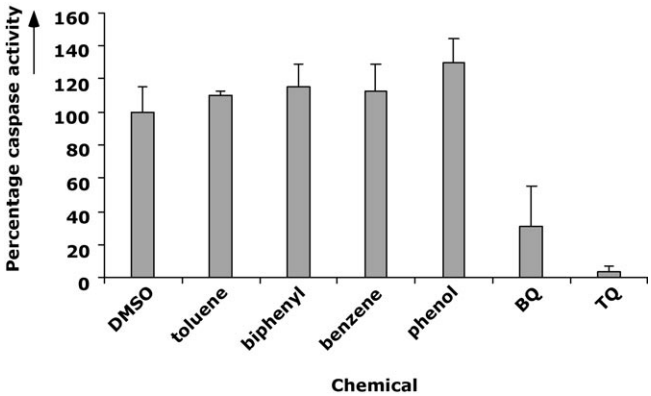


Figure 5. Inactivation of human caspase-3 by benzoquinone (BQ) and toluquinone (TQ). Human caspase-3 activity was monitored by the generation of fluorescent AMC compound from the cleavage of the caspase-3 substrate Ac-DEVD-AMC. ~10000 units of purified recombinant caspase-3 were preincubated with the caspase buffer alone or with the indicated chemicals before being assayed for caspase-3 activity as described in the Experimental Section. Biphenyl, benzene, phenol, and BQ were used at 100 μM; toluene and TQ were used at 1 mM. Each data point is an average of three independent experiments. Error bars show standard deviations.

organism. Benzene is likely the simplest member of this class.

One way that these chemicals might suppress apoptosis is by inhibiting caspases, the key executioner enzymes of apoptosis. In support of this hypothesis, we demonstrated that these benzenoid chemicals suppress ectopic cell killing in *C. elegans* caused by overexpression of the activated CED-3 caspase, independently of the CED-3 upstream activators. This in vivo evidence, combined with the observations that reactive quinone metabolites of these benzenoid chemicals can inhibit caspase activities in vitro, strongly suggests that these chemicals inhibit apoptosis by interfering with the functions of caspases.

It is worth noting that relatively high concentrations of quinone metabolites (100 μM) are needed to inhibit caspase activities in vitro (Figure 5);[5] this suggests that these chemicals alone are ineffective apoptosis inhibitors. Indeed, these benzenoid chemicals have very weak apoptosis inhibitory effects on wild-type animals (Figure 3).[5] However, in animals in which apoptosis is already compromised, such as the *ced-3*(*n2438*) mutant, these chemicals cause potent inhibition of apoptosis.

Our observations suggest that there is a nongenotoxic mechanism by which biphenyl, toluene, and benzene promote tumorigenesis, and might also explain why benzene is such a potent human carcinogen. The genotoxicity of benzene has been well characterized.[26] It has also been suggested that benzene acts through nongenotoxic mechanisms to promote leukemogenesis, possibly through suppression of apoptosis,[27,28] which is confirmed in our study. Therefore, if benzene simultaneously damages DNA and suppresses apoptosis, these two events can cooperatively promote cancer development.[4]

Toluene is a commonly used solvent found in many consumer products, including paints and adhesives. Toluene is considered "not classifiable" regarding its carcinogenic potential by the National Toxicology Program.[24] In vivo studies of the relationship between toluene exposure and cancer are limited and can be difficult to interpret because toluene exposure is often accompanied by exposure to other toxic chemicals including benzene.[24] Our observation that toluene suppresses somatic apoptosis in *C. elegans* and the evidence that toluene does not appear to be genotoxic[29] suggest that, unlike benzene, toluene might act exclusively as a tumor promoter; that is, it promotes tumor development through nongenotoxic mechanisms. Our data also suggest that the tumor-promoting activity of toluene might be easier to detect in mammals if toluene is administered in conjunction with genotoxic stress, or in situations in which DNA damage has already occurred.

Interestingly, not all benzenoid chemicals are capable of suppressing apoptosis in *C. elegans*. For example, mesitylene, which contains two additional methyl groups compared to toluene, fails to inhibit apoptosis in worms. These two additional methyl groups will most likely prevent the benzene ring from being metabolized to a reactive quinone. Alternatively, even if mesitylene can be metabolized into a quinone, these three methyl groups might cause steric hindrance, interfering with the interaction of the quinone product with its targets, such as the active site of caspases.[5]

In this study, we demonstrated that apoptosis assays in *C. elegans* can easily be used to investigate the effects of potential nongenotoxic carcinogens (and chemicals with structures closely related to those of known nongenotoxic carcinogens), and thus reduce the use of costly, time-consuming rodent models for such studies. More importantly, the powerful molecular genetic tools and available biochemical assays for cell death in *C. elegans*[21,23] will allow rapid identification of in vivo targets of chemical compounds, a major problem in drug discovery and toxicological studies. These have been used in our genetic and biochemical analyses that led to the identification of CED-3 caspase as the putative in vivo target of benzene and toluene. Therefore, compared to assays with recombinant enzymes, cell culture or rodents, the *C. elegans* model can provide a distinct advantage in facilitating the identification of in vivo targets of potential nongenotoxic carcinogens and other bioactive chemicals.

Whereas many standard assays are available to evaluate the genotoxicity of suspected carcinogens, the two-year rodent bioassay is currently the only standard assay to evaluate nongenotoxic carcinogens. The relatively short generation time (2.5 days per generation) and low maintenance cost of *C. elegans* makes it an attractive alternative animal model for evaluating the antiapoptotic activity of potential nongenotoxic carcinogens.

Experimental Section

Strains: *C. elegans* strains were maintained by using standard procedures.[30] The wild-type strain was N2. The following alleles were used in this study: LGIII: *ced-4*(*n2273*) and *ced-4*(*n1162*). LGIV: *ced-3*(*n2438*) and *ced-3*(*n2433*). LGX: *sml s111*.

Treatment of *C. elegans* animals with chemicals and phenotypic analyses of treated animals: Scoring of extra surviving cells and apoptotic cell corpses in *C. elegans* was performed as described.[31] Due to the high vapor pressure of some chemicals, fresh chemical solutions were made immediately before application. Biphenyl (CAS No. 92-52-4), benzene (CAS No. 1076-43-3), toluene (CAS No. 108-88-3), cyclohexane (CAS No. 110-82-7), mesitylene (CAS No. 108-67-8), and camphor (CAS No. 76-22-2) were dissolved in 100% soybean oil (Crisco). Gravid *C. elegans* adult animals were allowed to lay eggs for 1 h, so as to collect synchronized embryos. The adults were then removed, and oil solutions (2–3 mL) containing a chemical were spread onto each plate so that the surface of the nematode growth media (NGM) was completely covered. Worms live at the interface of the solid NGM media and the liquid oil. The resulting L3 and L4 larvae were scored for various defects including apoptosis inhibition. Oil solutions of toluene, benzene, mesitylene, and cyclohexane were used at a concentration of 400 mM, and solutions of biphenyl were used at a concentration of 200 mM unless otherwise noted.

Caspase activity assays using fluorogenic substrates: Human caspase-3 activity was determined by monitoring the cleavage of its fluorogenic substrate, Ac-DEVD-AMC. Purified human caspase-3[32] (1 μL, approximately 10,000 units) was pre-incubated on ice with the chemical (4 μL) dissolved in caspase buffer (20 mM PIPES, 75 mM NaCl, 2.5 mM EDTA, 0.1% CHAPS, 7.5% sucrose). BQ (CAS No. 106-51-4) and TQ (CAS No. 553-97-9) were obtained from Sigma. All chemical solutions were freshly made by dissolving the

chemicals in DMSO (100 mM) and then diluting this solution 1000 times in caspase buffer for a final concentration of 100 μM. In the "buffer alone" control, DMSO (1 μL) was diluted 1000 times in caspase buffer. After 15 min on ice, an aliquot of the reaction (5 μL) was diluted 100 times in the assay buffer (500 μL, caspase buffer with 10 mM DTT and 10 μM fluorogenic substrate). Substrate cleavage was monitored at a constant temperature of 25 °C by using 360 nm excitation and a 480 nm emission wavelengths and recorded once per second for 60 s. Under these conditions, fluorescence readings increased linearly for greater than 20 min.

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